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PATENT
Docket No.: **176/60792 (6-11415-868)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Mahin D. Maines	)	
Serial No. :	09/606,129	)	Examiner: D. Ramirez
Cnfrm. No. :	Unknown	)	Art Unit: 1652
Filed :	June 28, 2000	)	
For :	BILIVERDIN REDUCTASE FRAGMENTS AND VARIANTS, AND METHODS OF USING BILIVERDIN REDUCTASE AND SUCH FRAGMENTS AND VARIANTS	)	

DECLARATION OF MAHIN D. MAINES UNDER 37 C.F.R. § 1.132

I, MAHIN D. MAINES, pursuant to 37 C.F.R. § 1.132, declare:

1. I am inventor of the above-identified patent application.

2. I am currently Professor of Biochemistry and Biophysics at the University of Rochester Medical Center ("URMC"), Rochester, NY.

3. I received a B.A. in Biology from Ball State University in 1964, an M.S. in Chemistry from Ball State University in 1967, and a Ph.D. in Pharmacology from the University of Missouri in 1970.

4. I am presenting this declaration to demonstrate that biliverdin reductase ("BVR") binds to a number of protein kinases and, therefore, is likely to modify or regulate the activity of those protein kinases.

5. The protein kinases mediate eukaryotic cells' response to internal and external stimuli such as growth and differentiation factors, hormones, drugs, and chemicals. The cell signaling pathways that transduce stimuli depend on cascades of phosphorylation events and kinase activities. Biliverdin reductase ("BVR") has been identified as one kinase within the heme metabolic pathway, which sequentially converts heme to biliverdin and

biliverdin to bilirubin (via BVR). This heme metabolic pathway has been identified as a component of the cell signal transduction pathway.

6. I have previously demonstrated, in the above-identified application, that BVR is capable of binding to protein kinase C and that BVR as well as several fragments thereof are capable of regulating the activity of protein kinase C.

7. To demonstrate that BVR binds other human protein kinases, a yeast two-hybrid screening was performed as outlined below in paragraphs 8-10 using human BVR as the bait.

8. A bait vector capable of expressing human BVR was first constructed. The full length human BVR was amplified through PCR method using the following primers: (5' AATCCATGGCGAATGCAGAGCCCGAGAGGAA-3' and 5' GATGGATCCTCCTCCTCTTACTTCCTTG-3'). The PCR amplification was carried out using a wild type human BVR as a template under the following conditions: one cycle at 95°C for 50 seconds, 25 cycles of denature at 95°C for 50 seconds, annealing at 66°C for 50 seconds, and extension at 72°C for 70 seconds, finished with final extension for 10 minutes at 72°C. The PCR product was subcloned into a pGBT7 vector. To confirm that the bait could be used in the two-hybrid screening, it was transformed into yeast cells. Western blotting confirmed that the human BVR bait was expressed in the yeast cells, but the human BVR bait did not activate reporter genes in the transformed yeast cells nor did it show any obvious toxicity to yeast cells.

9. A Gal4-based yeast-two hybrid system 3 was purchased from Clontech. The Clontech system was used to screen a human kidney cDNA library using human BVR as bait. Screening procedures were carried out essentially in accordance with the manufacturer's instructions. The bait and prey libraries were simultaneously and briefly introduced into yeast strain AH109 using the standard Lithium acetate method, and the transformants were plated onto a SD defect medium. Approximately 17 million transformants were screened. Plasmids were isolated from yeast cells of Ade+/His+/MEL 1+ colonies and rescued by introduction into *E. coli*. The plasmids were analyzed by enzyme digestion. The plasmids were also transformed into yeast cells with bait to retest their phenotype. Thirty-one colonies were obtained for further analysis and, of these, fifteen were

isolated using stringent conditions (yeast plated on SD/-Ade/-His/-Leu/-Trp/X--Gal medium) and 16 were isolated under low stringency conditions (yeast plated on SD/-Leu/-Trp medium).

10. Following identification of clones which tested positive in the yeast two-hybrid assay, plasmid DNA was isolated and used in a PCR reaction along with Primer 719M2H and Primer 722M3M, which are specific for the plasmid pACT2. Primer 719M2H is a reverse primer having the sequence 5'-CTATTCGATGATGAAGATACCCCACCAAACCC-3' and Primer 722M3M is a forward primer having the sequence 5'-TGGCGAAGAAGTCCAAAGCTTCTGAATAAGCC-3'. The PCR products were isolated through agarose gel purification by using a Qiagen Gel Purification kit according to the manufacturer's instructions. The purified PCR products were sequenced to determine the predicted amino acid sequence of the protein that interacted with BVR in the yeast two-hybrid system. The sequencing reactions were cleaned by column purification by the Functional Genomics Center at the Core lab. Once the sequences of the inserts were obtained, the sequences were processed through the BLAST program found on the Nation Center for Biotechnology Information (NCBI) website. The BLAST program, used on its default settings, generated a listing of genetic data found in the NCBI database that match the sequence inserted into the program. Protein sequences were provided for some of the genetic data.

11. The protein sequences thus obtained were then examined in the MotifFinder (organized at the Supercomputer Laboratory, the Institute for Chemical Research, Kyoto University) search database to determine what motifs were responsible for binding with BVR. Default settings were used with MotifFinder, providing a search of both protein sequence (Swiss-Prot or PIR1) and structural (PDB) databases.

12. As a result of both the BLAST search described in paragraph 10 and the motif search described in paragraph 11, it was determined that at least one of the proteins identified is a protein kinase. Specifically, one of the identified proteins is the Goodpasture Antigen Binding Protein ("GABP"), whose sequence is reported in Genbank Accession No. NM_031361 (copy attached as Exhibit 1). As noted in the accession materials, the GABP itself is a protein kinase.

13. Having identified the GABP as a protein which binds to BVR, it is reasonable to hypothesize that BVR not only binds with the GABP but also modifies GABP activity. It is known that proteins which interact with kinases (and those proteins may themselves be kinases) often do so by binding to the kinase to regulate its activity (see, e.g., Maira et al., "Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane," Science 294(5541):374-80 (2001) (describing CTMP binding to carboxy-terminal region of protein kinase Bα to reduce activity of the kinase) (attached as Exhibit 2); Chen et al., "Isolation of protein kinase TAO2 and identification of its mitogen-activated protein kinase/extracellular signal-regulated kinase kinase bindng domain," J. Biol. Chem. 274(40):28803-7 (1999) (describing protein kinase TAO2 binding to and regulation of several mitogen-activated protein/extracellular signal-regulated kinase kinases) (attached as Exhibit 3); Sanchez-Olea et al., "Characterization of pICln phosphorylation state and a pICln-associated protein kinase," Biochim. Biophys. Acta 138(1):49-60 (1998) (describing the association of a protein kinase with pI_{Cln}, a ubiquitous cellular protein associated with chloride channel or a channel regulator) (attached as Exhibit 4)).

14. Based on the foregoing experimental work and the knowledge regarding kinase interactions, it is reasonable to expect the BVR not only binds to GABP but also regulates its activity.

15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: August 12, 2002

Mahin Maines

Mahin D. Maines